

# Molecular Characterization of Atlas 66-Derived Wheat Near-Isogenic Lines Contrasting in Aluminum (Al) Tolerance

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## Abstract

Aluminum (Al) toxicity is the major limiting factor for wheat growth in acidic soils. Genetic improvement of Al tolerance is one of the most cost-effective solutions to improve wheat productivity. The objective of this study was to characterize near isogenic lines (NILs) contrasting in Al tolerance derived from Atlas 66 in the backgrounds of Al-sensitive cultivars Chisholm and Century using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR). A total of 200 AFLP and 88 SSR primer pairs were screened and 12 markers (11 AFLPs and one SSR) were associated with Al-tolerance in NILs of at least one recurrent parental background. Among them, nine were linked to Al tolerance in the Chisholm-derived NILs, seven were associated with Al-tolerance in the Century-derived NILs, and three AFLPs derived from the primer combinations of pAG/mGCAG, pCAG/mAGC and pGTG/mGCG, and one SSR, Xwmc331 on chromosome 4D, associated with Al tolerance in NILs of both recurrent parental backgrounds. Those common markers across two backgrounds may be the major marker loci associated with Al-tolerance in Atlas 66 and could be useful for marker-assisted breeding to improve Al tolerance in wheat. In addition, evaluation of Al tolerance among different genotypes using hematoxylin stain and relative root growth revealed that Atlas 66 was more tolerant to Al stress than the NILs, therefore suggested that the Al-tolerant NILs might not carry all Al-tolerance loci from Atlas 66 and inheritance of Al tolerance in Atlas 66 is more likely multigenic.

**Key words:** aluminum (Al) tolerance, *Triticum aestivum* L., molecular marker

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the staple human food grains. Al toxicity in acidic soils limits the wheat production in many regions worldwide. Liming in acid soils can ameliorate Al toxicity, but it is expensive for transporting lime to destination field and ineffective in the subsoil. In some cases heavy lime appli-

cation may cause a deleterious effect on soil structure and alter the population composition of plant pathogens (Rao *et al.* 1993). Growing Al-tolerant cultivars is one of the best strategies for improving wheat productivity in acidic soils. Many efforts have been made to identify genetic sources of Al-tolerance in wheat (Aniol 1990; Berzonsky 1992; Carver *et al.* 1993). Genetics studies on wheat Al tolerance using the populations derived from the crosses between Al-tolerant and Al-

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sensitive cultivars indicated that a single dominant gene segregating for Al tolerance (Kerridge and Kronstad 1968; Riede and Anderson 1996; Raman *et al.* 2005). This gene, designated as either *Alt<sub>BH</sub>* or *Alt2*, was located on the long arm of chromosome 4D in different studies (Luo and Dovrack 1996; Riede and Anderson 1996). More recently aluminum-activated malate transporter gene (*ALMT1*) has been cloned and mapped on the same chromosome (Sasaki *et al.* 2004; Ma *et al.* 2005; Raman *et al.* 2005). However, several studies indicated that other genes might also be involved in Al tolerance in Atlas 66 (Iorczeski and Ohm 1977; Campbell and Lafever 1981; Anoil and Gustafson 1984; Berzonsky 1992; Pellet *et al.* 1996; Tang *et al.* 2002; Xiao *et al.* 2005). The analysis of wheat ditelosomic or chromosome deletion lines for Al tolerance suggested that chromosome arms 4DL, 5AS, 7AS and 2DL might also contain genetic factors controlling Al tolerance in Chinese Spring (Papernik *et al.* 2001; Ma *et al.* 2006).

Atlas 66 is an Al-tolerant cultivar released in the early 1940's from North Carolina, USA. Using Atlas 66 as the donor of an Al-tolerance gene, two sets of near isogenic lines (NILs) were developed in the backgrounds of Chisholm and Century by backcrossing (Carver *et al.* 1993). A further study suggested that one gene conferring fast root growth under Al stress was transferred from Atlas 66 into the tolerant NILs and that other genes with relatively minor effect in the recurrent parents might also influence Al tolerance (Johnson *et al.* 1997). Recently, Tang *et al.* (2002) reported that more than one gene might contribute to Al tolerance in Atlas 66. The analysis of gene expression profile of NILs under Al stress identified 25 functional genes in response to Al stress (Xiao *et al.* 2005). In this study, those Atlas 66-derived NILs are characterized with PCR-based molecular markers and the result will provide a useful information for understanding inheritance of Al-tolerance in Atlas 66 and genetic relationship among those NILs.

## MATERIALS AND METHODS

### Plant materials

Plant materials comprised Atlas 66 as the Al-tolerance

gene donor, two recurrent parents (Chisholm and Century) and their six BC<sub>3</sub>:F<sub>4</sub> NILs (Carver *et al.* 1993). Among those NILs, two Al-tolerant NILs (Chisholm-T1 and -T2) and one Al-sensitive NIL (Chisholm-S) were developed in the Chisholm background (Al-sensitive) and other two Al-tolerant NILs (Century-T1 and -T2) and one Al-sensitive NIL (Century-S) were derived from the Century background.

### Evaluation of Al tolerance

Wheat seeds were sterilized for 10 min using 0.5% NaOCl, rinsed in deionized water for 10 min, and germinated on wet filter paper in a petri dish at 23°C for 24 h before they were transferred to a polyethylene cup lined with nylon net on the bottom. The cups were floated on the surface of the nutrient solution in a 12-liter plastic container in a controlled growth chamber with a 16/8 h photoperiod regime at 24°C (day) and 20°C (night). The nutrient solution contained 5 mM CaCl<sub>2</sub>, 6.5 mM KNO<sub>3</sub>, 2.5 mM MgCl<sub>2</sub>, 0.1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.4 mM NH<sub>4</sub>NO<sub>3</sub> (Polle *et al.* 1978) and was replaced with fresh nutrient solution daily. Seedlings were grown in the nutrient-culture solution for two days, and then were transferred into the same nutrient solution with addition of 10 mg L<sup>-1</sup> AlK(SO<sub>4</sub>)<sub>2</sub>. The pH in the final nutrient solution was adjusted to 4.2 with HCl.

The primary roots from 10 seedlings of each genotype were measured daily for elongation rate after Al treatment. Relative root growth (RRG) was calculated as the ratio between mean root length of the Al treated seedlings and mean root length of the untreated control seedlings.

Al-stressed roots were also evaluated for hematoxylin stain score. After 24 h Al treatment, wheat seedlings were rinsed in deionized water for 1 h. The Al-stressed roots were submerged in a solution consisting of 0.2% (w/v) hematoxylin and 0.02% KIO<sub>3</sub> for 10 min. After rinsing with fresh water, roots tips were visually scored based on the degree of stain on the primary roots under a Nikon SMZ 1500 dissection scope (Nikon USA, Melville, NY, USA).

### AFLP and SSR analysis

Genomic DNA was extracted using the CTAB proce-

ture (Saghai-Marooft *et al.* 1984). AFLP analysis was performed as described by Bai *et al.* (1999). In brief, about 300 ng genomic DNA was double digested with *Pst* I and *Mse* I restriction enzymes. AFLP adapters for both restriction enzymes were then ligated to the restriction fragments. The ligated DNA was pre-amplified with a primer combination based on the sequences of the adapters without any selective nucleotide at the 3'-end. All PCR reactions were performed in an MJ PTC-100 thermocycler (MJ Research Inc., Watertown, Massachusetts, USA). Pre-amplification was performed for 30 cycles of 30 s at 94°C, 1 min at 65°C, and 1 min at 72°C. For selective amplification, all selective *Pst* I primers were labeled with [ $\gamma^{33}$ P] ATP for AFLP detection. The following touchdown thermal profile was used selective PCR amplification: One cycle of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C, 12 cycles in which the initial annealing temperature of 65°C was lowered by 0.7°C each cycle; and 23 cycles in which the annealing temperature was held constant at 56°C. AFLP-PCR products were separated on a 5% (w/v) denatured polyacrylamide gel followed by exposure to X-ray film (Kodak Biomax MR film, Rochester, New York, USA) for 2 to 3 d depending on intensity of signals. Two hundred combinations of *Pst* I and *Mse* I AFLP primers were screened for polymorphisms among the parents and NILs (Table 1).

Eighty-eight SSR primers were used to analyze polymorphisms among parents and NILs using a LI-COR automated sequencer (LI-COR Inc., Lincoln, NE, USA). For PCR, each 10  $\mu$ L of reaction contained 30 ng DNA, 1  $\times$  PCR buffer, 0.2 mM dNTP, 2 mM MgCl<sub>2</sub>, 1 pmol each of labeled and unlabeled SSR primers, and 1 unit of *Taq* polymerase. The following touchdown thermal profile was used for SSR amplification: 5 min of 95°C followed by five cycles of 45 s at 95°C, 5 min at 68°C, and 1 min at 72°C, in which the annealing temperature was lowered by 2°C in each cycle; then five more cycles

in which the annealing time was 2 min and the temperature was lowered by 2°C in each following cycle; 25 cycles in which the annealing temperature was held constant at 50°C. Five minutes at 72°C was used for final extension.

## Genetic similarity analysis

AFLP and SSR markers were visually inspected from radiograms and computer screen, respectively. Only unambiguous bands were scored and data were checked for accuracy twice. Each band was treated as a marker locus and scored as binary codes 1 and 0, where 1 represents presence of a band and 0 represents absence of a band. The matrix values estimating the number of AFLPs and SSRs shared between genotypes has been suggested as an appropriate estimator of relatedness under the assumption that the presence or absence of a discrete character in two or more genotypes results from same genetic changes (Skroch *et al.* 1992). Genetic similarity coefficients were estimated according to Nei and Li (1979). Cluster analysis was conducted using the unweighted pair-group method analysis (UPGMA) of NTSYSpc software, ver. 2.0 (Rohlf 1998), and presented as a dendrogram.

## RESULTS

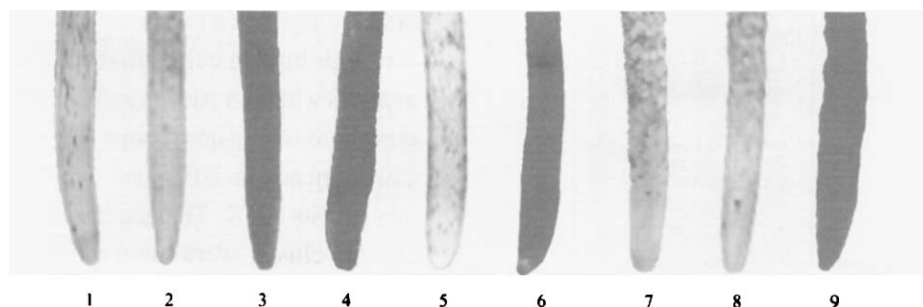
### Al tolerance in different wheat lines

Hematoxylin stain has been widely accepted as a rapid and reliable assay for Al tolerance among wheat cultivars (Polle *et al.* 1978). In the present study, tolerant and sensitive parents displayed contrasting staining patterns with very light stain on the root tips of Al-tolerant cultivar and heavy stain on the roots of Al-sensitive cultivars (Fig.1). Significant variation in staining pat-

**Table 1** Primer combinations for pre- and selective-amplification of AFLPs

Pre-amplification primers	
<i>Pst</i> I	GACTGCGTACATGCAG
<i>Mse</i> I	GATGAGTCCTGAGTAA
Selective amplification primers	
<i>Pst</i> I	pACT, pAG, pACTG, pAGT, pGCT, pACGC, pCAG, pCGT, pTAGC, pGTG
<i>Mse</i> I	mACC, mACGC, mAGC, mCAA, mCAC, mCACG, mCAG, mCAGT, mCAT, mCGAC, mCTA, mCTC, mCTG, mCTGA, mCTT, mGAC, mGCG, mTGC, mGCAG, mGTG

'p' and 'm' represent the pre-amplification primers of *Pst* I and *Mse* I, respectively.



**Fig. 1** Hematoxyline staining patterns of three wheat parents and their six NILs. Four-day-old seedlings were grown in the nutrient solution with  $10 \text{ mg L}^{-1} \text{ Al}^{3+}$  for 24 h. From 1 to 9 are wheat genotypes Chisholm-T1, -T2, -S, Chisholm, Atlas 66, Century, Century-T1, -T2, and -S, respectively.

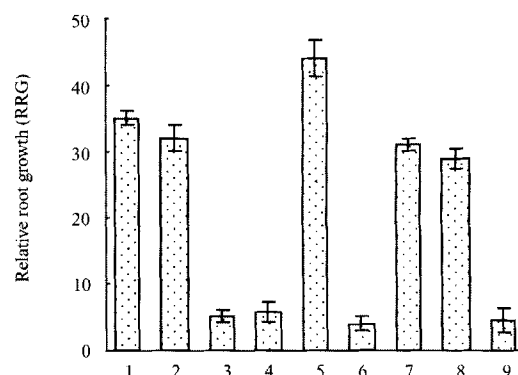
terns was observed among NILs. The root tips of the Al-sensitive NILs in both backgrounds, Chisholm-S and Century-S, were intensively stained as that for Al-sensitive parents, whereas the root tips of the Al-tolerant NILs (Chisholm-T1, Chisholm-T2, Century-T1 and Century-T2) had only light stain, which was a little darker than that for Atlas 66 (Fig.1).

Inhibition of root growth is a typical symptom of Al toxicity. Root elongation of all genotypes dramatically decreased after three days of exposure to  $10 \text{ mg L}^{-1} \text{ Al}^{3+}$  (Fig.2). However Atlas 66 and the four Al-tolerant NILs showed significant less reduction in root growth rate than recurrent parents and their Al-sensitive NILs under Al stress. After 3 days of Al stress, the root elongation of Atlas 66 was about 45% of the non-Al-treated control; Al-tolerant NILs had slightly lower RRG (around 35–38%) than Atlas 66, but substantially higher RRG than their two recurrent parents (5%).

## AFLP and SSR polymorphism among wheat lines

The possible combinations of 10 *Pst* I and 20 *Mse* I

selective primers (Table 1) were screened for all genotypes. A total of 174 (87%) primer combinations produced scorable AFLP banding patterns (Fig.3). Each primer pair produced 88 to 179 amplified bands with a

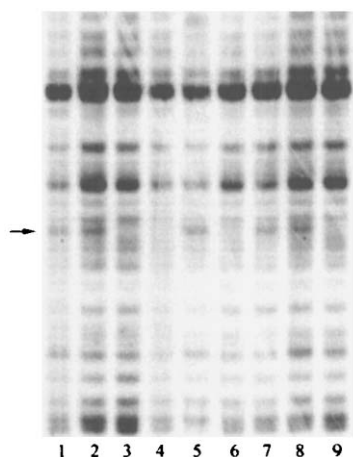


**Fig. 2** Effect of  $\text{Al}^{3+}$  on root growth of Chisholm-T1 (1), Chisholm-T2 (2), Chisholm-S (3), Chisholm (4), Atlas 66 (5), Century (6), Century-T1 (7), Century-T2 (8) and Century-S (9). Four-day-old seedlings were exposed to  $10 \text{ mg L}^{-1} \text{ Al}^{3+}$ . The length of the primary root from 10 seedlings was measured after 3 d of growth in the control and Al-treated seedlings. The percentage of relative root growth (RRG) was calculated as  $100 \times (\text{root growth of Al-treated seedlings} / \text{root growth of control seedlings})$ . Values are the means  $\pm$  SD from 10 replicated seedlings over two independent experiments.

**Table 2** Twelve combinations of primers that produced polymorphic markers between Al-tolerant and Al-sensitive NILs from two different genetic backgrounds of wheat

Primer combination	Markers in tolerance NILs from different background <sup>1)</sup>		Molecular size (bp)
	Chisholm	Century	
p-ACT/m-CTC	+(R)	-	130
p-ACT/m-CTT	-	+(R)	135
p-AG/m-GCAG	+(C)	+(C)	60
p-ACAG/m-AGC	+(C)	-	90
p-ACAG/m-CACG	+(C)	-	200
p-CAG/m-AGC	+(C)	+(C)	115
p-CAG/m-TGC	-	+(C)	110
p-CGT/m-CAC	+(C)	-	120
p-CGT/m-CTA	+(C)	-	520
p-CGT/m-TGC	-	+(C)	120
p-GTG/m-GCG	+(R)	+(R)	210
Xwmc331	+(C)	+(C)	131

<sup>1)</sup> C, coupling phase; R, repulsion phase; +, polymorphism between Al-tolerance and Al-sensitive NILs; -, no polymorphism.



**Fig. 3** Partial profile of AFLPs (pCAG/mAGC) exhibiting a polymorphic band (labeled with an arrow) co-migrating with Al-tolerance in coupling phase among wheat parental cultivars and near isogenic lines. Lanes 1 to 9 are Chisholm-T1, -T2, -S, Chisholm, Atlas 66, Century, Century-T1, -T2, and -S, respectively.

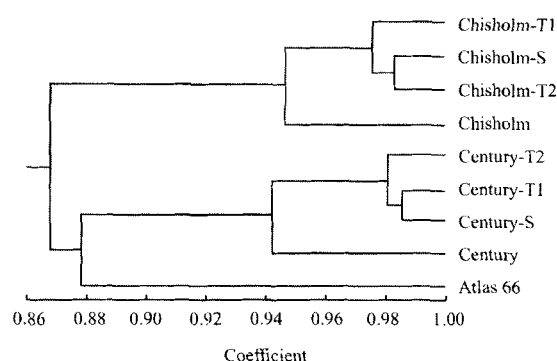
DNA fragment size range from 50 to 1 000 base pairs. Eighty-two primer combinations generated polymorphisms between Atlas 66 and recurrent parents and/or between their NILs contrasting in Al tolerance. Eleven primer pairs produced consistent polymorphisms between Al-tolerant NILs and Al-sensitive NILs in at least one background (Table 2). Eight of them produced polymorphic bands between contrasting NILs in Chisholm background, six produced polymorphic bands between contrasting NILs in Century background, and three (pAG/mGCAG, pCAG/mAGC and pGTG/mGCG) produced polymorphic bands between contrasting NILs in both backgrounds (Table 2 and Fig. 3). Most of polymorphic fragments were associated with Al tolerance in coupling phase in the NILs, and only 3 polymorphic fragments were associated with Al tolerance in repulsion phase in the NILs.

Eighty-eight SSR primer pairs were screened for polymorphism among three parents and six NILs. Only Xwmc331 showed polymorphism between parents and NILs contrasting in Al tolerance (Table 2). Xwmc331 has been previously located on chromosome 4DL (Somers *et al.* 2004) and showed the same pattern as the three AFLP markers among the genotypes analyzed in the study.

### Genetic similarity analysis of NILs

Haplotyping of various genotypes with molecular mark-

ers may provide a reasonable estimate of genetic relationships among cultivars (Bai *et al.* 2003). UPGMA analysis with 428 AFLPs and 20 SSRs clustered 9 genotypes into two major groups (Fig. 4). Group I included Chisholm and its NILs, and Group II consisted of Century and its NILs. The genetic similarities among NILs in each cluster were high, varying from 0.98 to 0.99 although the NILs in each cluster had contrasting responses to Al. The genetic similarities between the recurrent parents and its NILs were around 0.94. Atlas 66 was closer to Century than Chisholm, but was farther to Century's NILs than Century to the NILs.



**Fig. 4** UPGMA dendrogram depicting patterns of genetic similarity based on 428 AFLP and 20 SSR marker alleles among Atlas 66, recurrent parents and their NILs.

## DISCUSSION

AFLP and SSR have been widely used in haplotyping and molecular mapping of numerous crops (Bai *et al.* 2003). In the current study, AFLP and SSR were used to characterize the genetic relatedness between Al-tolerant NILs and to identify molecular markers linked to Al tolerance using RILs previously developed (Carver *et al.* 1993). The result will provide useful information for effective use of the RILs in breeding and genetic researches. Among 200 AFLP primer combinations tested, majority of primer pairs produced about 100 scorable fragments per primer pair. The polymorphic rate among the parental cultivars and six NILs was 10.8%, relatively lower than previous reports of 12.6% (Bai *et al.* 1999), and 13.2% (Barrett and Kidwell 1998) in the inbred recombinant lines of wheat. This is due to that only three cultivars were included and majority of genomes of the NILs were identical in this study. However, polymorphic rate

of AFLP was much higher than that of SSR. Only one out of 88 SSR showed polymorphism both between contrasting parents and between NILs. Therefore AFLP marker is a useful marker system for high resolution mapping of QTL for Al tolerance.

Phenotypic data indicated that Al-tolerance gene (s) from the donor parent Atlas 66 were transferred to the Al-tolerant NILs, which agreed with a previous report (Johnson *et al.* 1997). Further analysis with AFLP and SSR markers on all NILs and their parents showed that nine and seven polymorphic fragments co-migrated with the Al-tolerant NILs of Chisholm and Century, respectively. Only four of these polymorphic fragments were common among four resistant NILs in both backgrounds (Table 2), indicating that these four markers may be closely linked to an Al-tolerance gene from Atlas 66, and Al tolerance in those Al-tolerant NILs is at least controlled by one common gene/QTL. However, those NILs showed only partial tolerance to Al toxicity especially when RRG was examined. Although the roots of the tolerant NILs in both backgrounds grown much faster than that of sensitive genotypes, RRG is significant lower than that for Atlas 66. The results indicated that those NILs only carry partial resistance from Atlas 66, most likely one major QTL from Atlas 66, which is also coincident with the report by Tang *et al.* (2002). Recently, a wheat gene encoding Al-activated malate transporter (ALMT1) was isolated (Sasaki *et al.* 2004) and located in the major QTL region on chromosome 4DL (Ma *et al.* 2005; Raman *et al.* 2005). Atlas 66 also has this gene on 4DL, and the SSR marker Xwmc 331 that was common across all Al tolerant NILs has been mapped on the 4DL QTL region previously (Ma *et al.* 2005). Therefore, the Al tolerance in all four NILs is most likely conferred by the major QTL on 4DL that harbors the ALMT1 gene.

Raman *et al.* (2005) suggested ALMT1 is the gene fully responsible for Al tolerance in wheat as previously proposed (Riede and Andersom 1996). However, some other findings were not fully agreement with this assumption and suggested that multi-gene mechanisms might be involved in Wheat Al tolerance (Pellet *et al.* 1996; Gallego and Benito 1997; Ahn *et al.* 2004; Xiao *et al.* 2005; Ma *et al.* 2006). Beside chromosome 4DL, three regions on 5AS, 7AS and 2DL were identified to contribute Al-tolerance (Papernik *et al.* 2001; Ma *et al.*

2006). Interestingly, four Al-tolerant NILs that carry the 4DL major QTL only inherit partial Al tolerance from Atlas 66 in the present study, suggesting that other gene/QTLs for Al tolerance may also exist in Atlas 66 and has not been transferred into the NILs. Therefore, the QTL on 4DL may be the only QTL responsible for Al-tolerance in Atlas 66 and other QTLs with minor effects on Al tolerance may also be responsible for a high level of Al tolerance in Atlas 66.

Molecular marker profiles may provide the best estimate of genetic relationships among genotypes (Bai *et al.* 2003). Cluster analysis on the base of AFLP and SSR markers clearly separated the NILs into two backgrounds, Century and Chisholm. That similarity between Atlas 66 and its NILs was much lower ( $< 0.88$ ) than that between recurrent parents and their NILs (0.95) indicated that 95% genetic background of a NIL is identical to the recurrent parent after three backcrosses, which agreed with a theoretical estimate of a backcross selection scheme (Carver *et al.* 1993). However, 5% of dissimilarity between recurrent parent and Al tolerant NILs indicated that some other portions of genomes in the NILs still differ from recurrent parents. Therefore, genetic difference between the recurrent parents and their Al-tolerant NILs may still be affected by genetic background in some degrees. Fortunately, genetic similarity between Al tolerance and sensitive NILs was much higher than that between recurrent parents and their Al-tolerant NILs, especially Century-T1 vs Century-S and Chisholm-T2 vs Chisholm-S, therefore they are better (-99%) choice of NILs for genetic research.

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